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In vitro Differentiation Mode of Pancreatic Cells into Multiple Neuronal Phenotypes

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Abstract: The *in vitro* differentiation features of HPNE cells into neural cells were investigated. In this study, HPNE cells were pre-cultured in defined medium, and then induced by ITS treatment (insulin-transferrin-selenium). After a transient ductal stage, HPNE cells present neural cell morphology. A series of neural markers were detected in terminally transformed HPNE cells. During HPNE transdifferentiation, the components of Notch signaling were elaborately modulated. These findings indicate that HPNE cells have the differentiation potential into neural cells.

Key words: HPNE cells, *in vitro* differentiation model, ITS treatment, neural

INTRODUCTION

The nervous system is divided into two parts: the central nervous system consisting of the brain and spinal cord, and the peripheral nervous system consisting of cranial and spinal nerves along with their associated ganglia. Worldwide, nervous system injuries are affecting over 90,000 people every year (Lenzlinger *et al*., 2005). However, the central nervous system is, for the most part, incapable of self-repair and regeneration. Therefore, nerve regeneration from in vitro system is becoming a very promising research field for the discovery of new ways to cure nerve dysfunction.

To achieve this goal, we must first understand the gene regulatory network that directs neural cell growth and differentiation. During the past twenty years, a large group of transcription factors and growth factors involved in neural development and neurogenesis have been identified (Yun *et al*., 2010). In the early neural specification, morphogen BMP4 gene could trigger downstream signaling like Smad proteins etc, to determine whether ectoderm cells are committed into neural or epidermis fate (Aberdam *et al*., 2007). During CNS compartmentalization, the polar distribution of WNT, FGF, RA signaling and Hox genes play important roles in establishing regional cell identity (Steventon *et al*., 2005). For Dorsal-Ventral patterning, graded SHH expression induces some of homeobox genes (e.g., Nkx2.2, Nkx6.1) while inhibiting others (e.g., Pax6, Dbx2) (Lendahl *et al*., 1997). From neural crest progenitors to neuroblast terminal differentiation, a cascade of transcription factors are now recognized to be critical regulators, including Ngn, Pax, MBP, MAP, Beta-tubulin, GFAP, etc., (Rogers *et al*., 2009). In addition, neural development is even more complicated than we speculated, in which multiple processes are involved, like neural migration, Axonal guidance, Synaptogenesis, etc.

Trans-differentiation of one cell fate to another has been intensively studied in order to produce useful tissues for regenerative medicine. It has been well evidenced by many researches with the possibility of transdifferentiation into neural phenotype from other cell types. Various sources and strategies are now broadly employed in neural studies. ES cells were one of the ideal candidates on neural differentiation for its pluripotency and unlimited self-renewal (Schwarz and Schwarz, 2010). Bain *et al*. (1996) firstly showed that retinoic acid treatment can induce neural differentiation on ES cells, with a high proportion of the resulting cells expressed neural markers and had neural properties. In particular, NSCs from embryo CNS are considered to be more powerful for its advantage - committed neural lineage (Hsu *et al*., 2007). Later, Adult NSCs were found in SVZ and SGL of hippocampus, which under FGF-2 stimulation were capable of differentiating into astrocytes, oligodendrocytes and neurons (Lowry and Richter, 2007). Although stem cells have such advantages, their application has been impeded by a number of issues like limited sources, tumorigenicity, ethical consideration, long term maintenance, etc. Therefore, many studies are seeking to find non-NSCs as secondary choices, like bone marrow stem cells (BMSCs), umbilical cord stem cells (UCSCs), etc., (Egusa *et al*., 2005; Herranz *et al*., 2010).

In the present study, we investigate the molecular mechanism underlying neural differentiation using a human primary pancreas acinar cell line HPNE cell. HPNE cell is immortalized by hTERT cDNA (Lee *et al*., 2003), in which Notch and Nestin genes are active. Both are precursor cell maker in cellular differentiation, especially in nervous and endocrine

Table 1: The primer sequence and product size for RT-PCR

Gene name	Forward primer	Reverse primer Size	(pbs)
Beta-actin	CATCGAGCACGGCATCGTCA	TAGCACAGCCTGGATAGCAAC	211
Notch1	ACATCCCGCCCCGGGGGCTG	CTTCGCTGGTGTACCAGTGC	141
Notch2	AATGTCCGTGGCCCAGATGG	TCTGCACCTGCATCCAGGAG	242
Hes1	CACTCGGCTGCTCGGCCACC	CGCGCCCCCGGGGATGGGCA	175
Ngn1	AACTTGAACGCGGCCCTGGA	CAGATGTAGTTGTAGGCGAA	223
Ngn2	CACGTTCCCCGAGGACGCCA	CAACACTGCCTCGGAGAAGA	154
Ngn3	CAACCTCAACTCGGCACTGG	ATGTAGTTGTGGGCGAAGCG	105
MAP	CTGAAGAAACAGCTAATCTG	CTGATTTTAGGTATGGCAAA	301
MBP	TGCCCCCAGACGCACGTGGG	GGAGGTGAGAGAAGGACAGG	230
GFAP	GTGGTACCGCTCCAAGTTTG	TCCAGGGACTCGTTCGTGCC	156
Beta-tubulin	GAGTGAGAGCTGTGACTGCC	AATTCATGATCCGGTCCGGG	128

(Lee *et al*., 2005). We innovatively established a neural transdifferentiation model via metaplasia and differentiation induction method. A series of neural markers were detected in HPNE cells after induction, including Beta-tubulin (Falconer *et al*., 1994), MAP2 (Shafit-Zagardo and Kalcheva, 1998), MBP (Fitzner *et al*., 2006), GFAP (Liedtke *et al*., 1996), etc. While Notch signaling associated genes were differentially regulated, like Ngn1, Ngn2, Notch1, Notch2, Hes1, etc. These results suggest that HPNE cells have the potential to differentiate into neural phenotype through multiple cellular events.

MATERIALS AND METHODS

The experiments for this study were carried out during the academic session 2007-2010 at the Research institute for Children in Children's Hospital at New Orleans, US.

Cell lines and chemicals: A human pancreatic Acinar adenocarcinoma cell line (HPNE) was purchased from ATCC and maintained according to ATCC's recommendation. Mouse monoclonal anti Ngn3 antibody and Rabbit polyclonal anti Beta-tubulin antibody were purchased from Abbiotec (San Diego, CA). Fluorescence conjugated secondary antibodies were purchased from SouthernBiotech (Birmingham, AL). DAPI was purchased from Invitrogen (Carlsbad, CA). All other chemicals were purchased from Sigma (St. Louis, MO).

Metaplasia and differentiation induction:
Metaplasia induction: HPNE cells are cultured in medium D, and treated with 5uM sodium butyrate and 5mM 5-aza-2'-deoxycytidine for 2~3 days (Lee *et al*., 2005).

Differentiation induction: Ductal HPNE cells are treated with 0.05% trypsin at 25ºC for 90 sec, then cultured in defined SFM(DME/F12) containing 17.5 mM glucose, 1% BSA and insulin- transferrin- selenium (Hardikar *et al*., 2003).

RNA isolation and RT-PCR: Total RNA was isolated from HPNE cells using the Trizol reagent (Invitrogen, Carlsbad, CA). The isolated RNA was treated with DNase I (Promega, Madison, WI) and precipitated using isopropanol. A total of 2 μg of the isolated RNA was processed for reverse transcription using a cDNA synthesis kit (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. PCR conditions were 95ºC for 5 min, followed by 40 cycles of denaturation at 95ºC for 30 sec, annealing at 55ºC, and extension at 72ºC for 30 sec. The PCR products were separated on 2% agarose gel. The PCR primer pairs and the product sizes are listed in the Table 1.

Immunofluorescence and fluorescence microscopy: The cells were fixed in PBS containing 2% paraformaldehyde and permeabilized in PBS containing 0.2% Triton X-100 and 0.5% BSA for 15 min at 4ºC. Cells were incubated in blocking solution (0.5% BSA and 2 mg/mL human IgG) for 30 min at room temperature. Staining was conducted in blocking solution with antibodies followed by secondary antibodies conjugated to FITC or PE, respectively. The fluorescent signals were detected by a fluorescence microscope and the nucleus was stained with 4', 6-diamidino-2-phenylindole (DAPI). The pictures were captured using a SPOT digital image system (Diagnostic instruments, Sterling Heights, MI).

RESULTS

HPNE cell lines exhibit morphology of neural differentiation: To develop a neural transdifferentiation, we use human pancreas nestin positive acinar cell line - HPNE cells. After exposed to sodium butyrate and defined medium, HPNE cells underwent metaplasia from acinar to ductal status (Fig. 1). Then the following ITS treatment leads to the formation of dendritic tree - typical neural morphology. This conclusion was further proved by next assays of gene expression pattern

Notch signal pathway is involved during HPNE transdifferentiation: Notch signal pathway was shown in the central role of controlling the transition into or exit

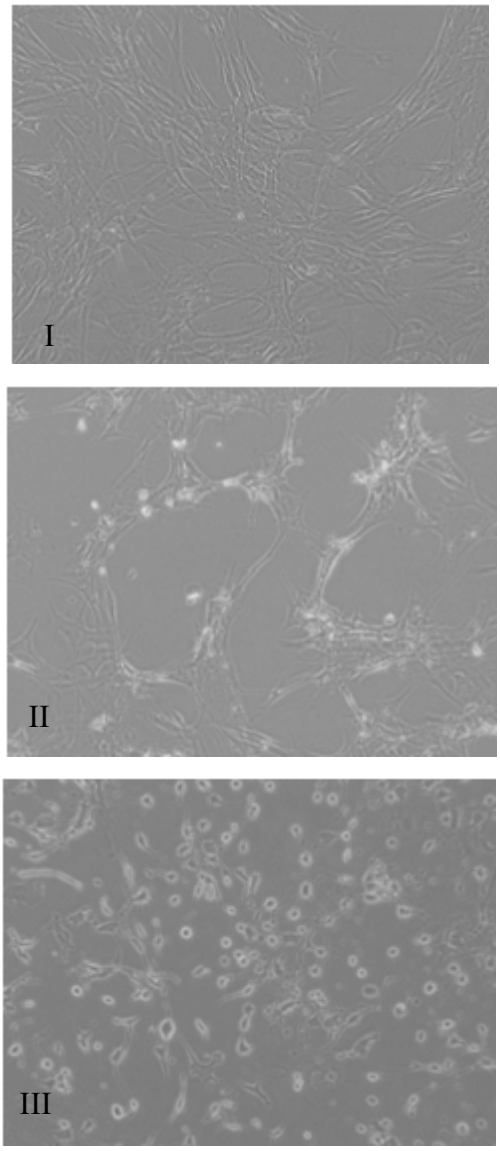

Fig. 1: The transition of HPNE cells during transdifferentiation. I. Normal stage, II. Ductal stage, III. Neural stage

ductal cell fate. And normal HPNE cells have minimal endogenous mRNA expression of Notch and other components. Here, we found that Notch1, Notch2 and Hes2 expression were first largely decreased, and then slightly increased in our HPNE transdifferentiation model (Fig. 2A). It suggests that a transient ductal stage occurred before HPNE cells eventually convert into neural phenotype. As we know, NGN protein family is the inhibitor of Notch signal pathway. In consistency with the changes of Notch proteins, three of Ngn protein family members Ngn1, Ngn2 (Ma *et al*., 1999) and Ngn3 (Jensen *et al*., 2000) were detected with slight increase (Fig. 2B).

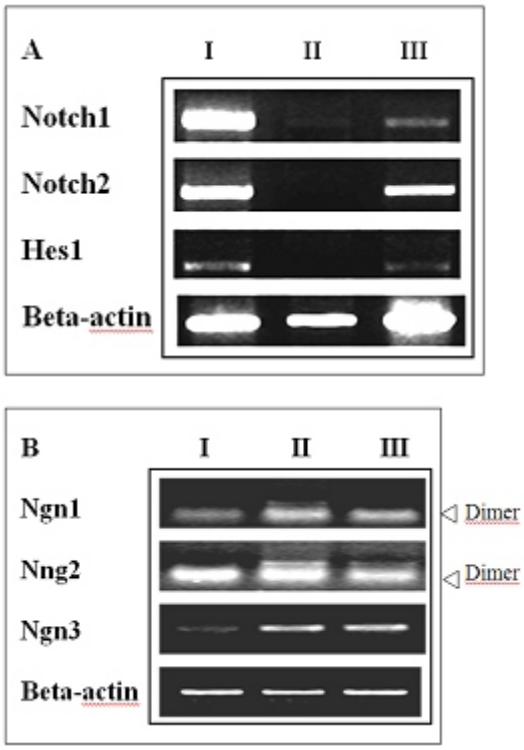

Fig. 2: The Notch signal tranduction during HPNE transdifferentiation. A. Notch family gene expression; B. Ngn family gene expression

Neural Markers are detected in differentiated HPNE cells: A lot of molecular markers were screened in our neural transdifferentiation model. In Fig. 3A, we have detected mRNA expression of four neural markers, respectively for Beta-tubulin, MAP2, MBP, and GFAP. It was found that only MBP protein expressed in normal HPNE cells while three other proteins were still absent before induction. Immuno-fluorescence staining further confirmed that Beta-tubulin expression was induced in the cytoplasm of HPNE cells (Fig. 3B).

DISCUSSION

Neural differentiation is of great significance in neural developmental studies as well as regenerative therapy for neural injuries. To elucidate the molecular mechanisms underlying this critical biological event, we established a novel neural transdifferentiation model using pancreatic nestin positive cell line HPNE cells with metaplasia induction and ITS treatment. In our system, Notch signal pathway was found to be modulated correspondingly to specific stages of HPNE cells. And a subset of neural markers was detected during HPNE transdifferentiation, like Beta-tubulin, MAP2, MBP, GFAP, etc.

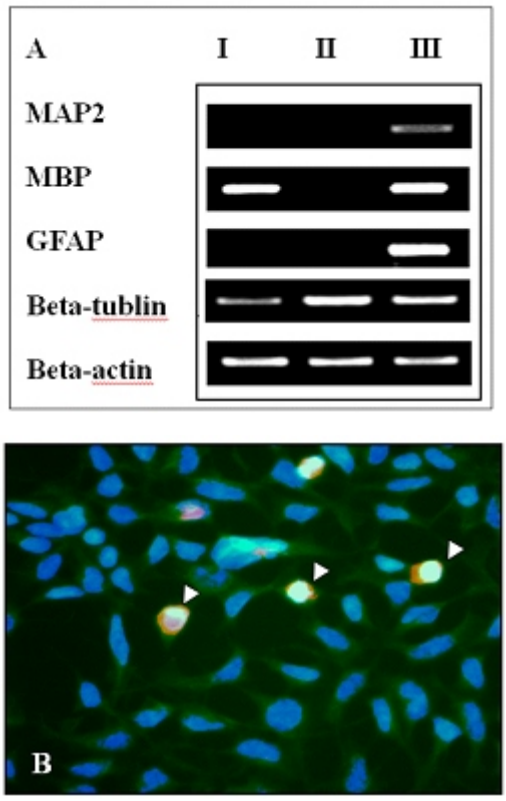

Fig. 3: The neural feature of HPNE cells transdifferentiation. A. Neural marker expression; B. Ngn3 (Red)/Beta-tubulin (Green) double staini

HPNE cells are primary pancreatic nestin positive cell line, which have the potential to develop an endocrine phenotype in pancreatic origin. Interestingly, a rare phenomenon was observed that HPNE firstly present a polygonal morphology after metaplasia induction, then transform into dendritic morphology. These results suggested that multiple stages of transdifferentiation processes in HPNE cells control the switch between ductal and neural cell fate.

Notch signaling plays an important role in neural development, which engages a number of bHLH Transcription Factors, like Mash, HES, Neurogenin, etc., (Bolos *et al*., 2007). Here, we found that the components of Notch signal pathway are under elaborate modulation through HPNE cell fate transitions. At the first transition from acinar to ductal cell fate, Notch proteins increased while Notch negative regulators Neurogenin proteins were absent; At second transition from ductal to neural cell fate, Notch proteins gradually decreased while Neurogenin proteins were then induced. These results indicate that Notch signaling was promoted for ductal specification while inhibited for neural specification.

Molecular mechanisms of neural development are very intricate and involve a cascade of transcription regulations. Diverse neural markers were detected during HPNE transdifferentiation, like Beta-tubulin, MAP2, MBP, GFAP, etc. Beta-tubulin is one of the earliest markers to signal neural commitment in primitive neuroepithelium. MAP2 is a marker of neuronal differentiation as a neuron-specific protein that stabilizes microtubules in the dendrites of postmitotic neurons. MBP is myelin basic protein, the early oligo-dendroglial marker. GFAP is glial fibrillary acidic protein, a marker for glial cells. Their expression patterns indicate that miscellaneous differentiated HPNE cells arise from a uniform origin.

Part of the reason lies in the Notch signaling, which controls a binary fate decision during neural development. While the early (neural versus epidermal) fate decisions mainly involve an inhibitory effect of Notch on the neural fate, late fate decisions (choice between different subtypes of neural cells) have been proposed to involve a binary switch activity whereby Notch would guide one fate while inhibiting another. Therefore, it is most likely that HPNE cells might be committed to diverse cell fates via a well-toned notch signaling.

CONCLUSION

This data from this study suggest that multiple step treatments could convert HPNE cells from pancreatic acinar cell type into neural cell type, accompanied with the expression of specific neural markers as well as suppression of Notch signal pathway.

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